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Regulation of Plasminogen Activation by Components of the Extracellular Matrix†

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ABSTRACT: The kinetics of activation of Glu-plasminogen (Glu-Pg) and Lys_{77}-Pg by two-chain recombinant tissue plasminogen activator (t-PA) were determined in the presence of isolated protein components of the extracellular matrix (ECM) and compared to activation in the presence of fibrinogen and fibrinogen fragments and in the absence of added protein. Several ECM protein components were as effective as fibrinogen fragments at stimulating Pg activation. Stimulation of Glu-Pg activation resulted from both a decrease in K_m and an increase in V_{max}, whereas stimulation of Lys_{77}-Pg was due primarily to increases in V_{max}. The most effective stimulators of activation were basement membrane type IV collagen and gelatin which resulted in a 21- and 55-fold increase, respectively, in the k_{cat}/K_m of Glu-Pg (relative to a 10-fold increase observed with fibrinogen fragments). Amidolytic activity of t-PA was also enhanced up to 12-fold by ECM proteins. However, plasmin amidolytic activity was unaffected by the presence of added proteins. These data suggest that several ECM-associated proteins can enhanced the activation of Pg in the absence of fibrin.

Plasminogen (Pg)[1] is the circulating zymogen form of the serine proteinase plasmin (Pm). Conversion of Pg to Pm is catalyzed by a number of plasminogen activators [for a review, see Castellino (1981)]. Activation of Pg by tissue plasminogen activator (t-PA) occurs very slowly in systems containing only Pg and t-PA (Hoyalerts et al., 1982). However, several studies have demonstrated that Pg activation is stimulated when the zymogen, activator, or both are immobilized by binding to a macromolecular protein surface such as fibrin (Ranby, 1982) or CNBr fragments of fibrinogen (Nieuwenheuizen, 1983).

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[1] Abbreviations: Pg, plasminogen; t-PA, tissue plasminogen activator; ECM, extracellular matrix; TSP, thrombospondin; HRGP, histidine-rich glycoprotein; VLK-pNA, D-Val-Leu-Lys-*p*-nitroanilide; IPR-pNA, D-Ile-Pro-Arg-*p*-nitroanilide; BSA, bovine serum albumin; FN, fibronectin; LN, laminin-nidogen; Pm, plasmin.

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In addition to its role in fibrinolysis, Pm is implicated in a variety of physiological and pathological processes involving modification of the extracellular matrix (ECM) including embryogenesis (Strickland et al., 1976), inflammation (Reich et al., 1978), wound healing (Highsmith, 1981), and neoplasia (Ossowski & Vassalli, 1978). The role of Pm in extracellular matrix modification suggests that conversion of Pg to Pm occurs under conditions which differ from those in the intravascular space. Previous studies have demonstrated that proteins other than fibrin have a potentiating effect on Pg activation by t-PA. Silverstein et al. (1985) reported that binding of Pg and t-PA to thrombospondin (TSP) and histidine-rich glycoprotein (HRGP) enhanced Pg activation. Intact extracellular matrices synthesized in vitro by endothelial cells also bind Pg and stimulate activation by t-PA (Knudsen et al., 1986).

The ECM is composed of a complex variety of macromolecules with the potential to influence Pg activation. We have determined the effect of isolated ECM protein components on the kinetics of Pg activation by t-PA. Our studies indicate that several ECM-associated proteins, most notably type IV collagen, stimulate Pg activation in the absence of fibrin.

Materials and Methods

Materials

D-Val-Leu-Lys-*p*-nitroanilide (VLK-pNA, or S2251), D-Ile-Pro-Arg-*p*-nitroanilide (IPR-pNA, or S2288), and human fibrinogen were purchased from Helena Laboratories, Beaumont, TX. Fibrinogen was rendered Pg-free by chromatography on L-lysine–Sepharose. CNBr fragments of fibrinogen were generated according to the method of Blomback et al. (1968). Human collagen types I, III, IV, and V and bovine serum albumin (BSA) were products of Sigma Chemical Co., St. Louis, MO. Purity of collagens was assessed by electrophoresis on 7.5% SDS–polyacrylamide gels under reducing conditions (Laemmli, 1970). Gelatin was produced by thermal denaturation of the corresponding collagen type by heating for 20 min at 60 °C (Murphy et al., 1981). Fibronectin (FN) was purified from pooled plasma according to the method of Vuento and Vaheri (1979). Urokinase was purchased from Calbiochem, San Diego, CA, and coupled to CNBr-activated Sepharose 4B according to the method of Cuatrecasas et al. 1968). Laminin–nidogen complex (LN) was the generous gift of Dr. Peter Yurchenco of the University of Medicine and Dentistry of New Jersey, and two-chain recombinant tissue plasminogen activator (t-PA) was supplied by Dr. Henry Berger at Wellcome Research Laboratories, Research Triangle Park, NC. All other chemicals were of reagent-grade quality.

Methods

Proteins. Pg was purified from human plasma by affinity chromatography on L-Lys-Sepharose (Deutsch & Mertz, 1970). Pg isoforms 1 and 2 were separated by affinity chromatography on concanavalin A–Sepharose (Gonzalez-Gronow & Robbins, 1984). Affinity chromatography purified form 2 was utilized for all experiments. Pm was generated by incubating 100 μg of Pg with 100 μL of urokinase–Sepharose in 10 mM Hepes, pH 7.4, for 1 h at 25 °C followed by centrifugation to remove the resin. Lys_{77}-Pg was prepared by limited proteolysis of Glu-Pg with Pm in a molar ratio of 10:1 for 30 min at 25 °C followed by chromatography on pancreatic trypsin inhibitor–Sepharose to remove Pm (Castellino & Powel, 1981). Protein concentrations were determined spectrophotometrically at 280 nm using an $A_{1\%/1cm}$ value of 16.8 and molecular weights of 92000, 83000, and 81000 for Glu-Pg, Lys_{77}-Pg, and Pm, respectively (Castellino, 1981).

Kinetics of Pg Activation. Coupled assays were used to evaluate the initial rate of Pg activation by t-PA by monitoring the amidolytic activity of generated Pm (Wohl et al., 1980). Glu-Pg (0.015–1.0 μM) or Lys_{77}-Pg (0.03–0.40 μM) was incubated at 37 °C in 10 mM Hepes, pH 7.4, with the Pm substrate VLK-pNA (0.3 mM final concentration). Chloride was omitted from all buffers since it has been demonstrated that activation of Glu-Pg by t-PA is inhibited by high concentrations of Cl^- (Urano et al., 1988). Activation of Pg was initiated by addition of 0.55 nM (20 IU/mL) t-PA, and the Pm hydrolysis of VLK-pNA was monitored continuously at 405 nm. Alternatively, reactions were terminated by addition of 50 μL of 50% acetic acid, and the absorbance at 405 nm was determined. Initial velocities (v_i) were calculated from the slope (b) of plots of A_{405} vs time^2 by using the equation $v_i = b(1 + K_m/S_0)/\epsilon k_e$ (Wohl et al., 1980) where K_m is the apparent Michaelis constant of VLK-pNA hydrolysis by Pm (0.3 mM), k_e is the empirically determined catalytic rate constant for Pm hydrolysis of VLK-pNA [3.2×10^4 M min^{-1} (mol of Pm)$^{-1}$], and ϵ is the molar extinction coefficient of *p*-nitroanilide at 405 nm (8800 M^{-1} cm^{-1}, Erlanger et al., 1965).

To determine the effect of ECM proteins on Pg activation kinetics, Pg was preincubated with 0.5 μM protein in a 250-μL final volume for 1 h at 37 °C. After incubation, Pg activation was monitored as described above. For reactions containing collagen, buffers contained 0.25 M glucose to prevent collagen precipitation (Terato et al., 1976). Activation reactions were also performed in the presence of fibrinogen (0.5 μM), CNBr fibrinogen fragments (50 μg/mL), or BSA (0.5 μM). Data were analyzed by the method of Lineweaver and Burk (1934) to evaluate kinetic constants.

Effect of ECM Proteins on Amidolytic Activity of Pm. Amidolytic activity of Pm in the presence of proteins was determined by incubating Pm (11 nM) with ECM protein, fibrinogen, or BSA (0.5 μM) or CNBr fibinogen fragments (50 μg/mL) at 37 °C in 10 mM Hepes, pH 7.4, followed by addition of VLK-pNA (0.3 mM). Hydrolysis of VLK-pNA was monitored continuously at 405 nm, and the amount of *p*-nitroanilide released was quantitated by using an extinction coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1965).

Effect of ECM Proteins on Amidolytic Activity of t-PA. Amidolytic activity of t-PA in the presence of proteins was determined by incubating t-PA (0.55 nM) with ECM protein, fibrinogen, or BSA (0.5 μM) or CNBr fibrinogen fragments (50 μg/mL) at 37 °C in 10 mM Hepes, pH 7.4, followed by addition of IPR-pNA (0.3 mM). After incubation for 40–120 min at 37 °C, the reaction was terminated by the addition of 50 μL of 50% acetic acid, and substrate hydrolysis was quantitated by measuring the absorbance at 405 nm.

Results

Kinetics of Pg Activation in the Presence of ECM Protein Components. Activation of Glu-Pg and Lys_{77}-Pg by t-PA in the presence of ECM-associated proteins was studied by using the Pm substrate VLK-pNA. During the course of the investigation, we found that Lineweaver–Burk plots of activation initial rates with Glu-Pg in the presence of fibrinogen fragments and gelatins I and IV were nonlinear at Glu-Pg concentrations greater than 0.05 μM due to apparent substrate inhibition. Therefore, for evaluation of kinetic constants in the presence of these proteins, Glu-Pg concentrations of less than 0.05 μM were used. Kinetic analysis for these data using the method of Eadie-Hofstee (Eadie, 1942; Hofstee, 1959) does not result in significant changes in kinetic parameters. Ac-

Table I: Kinetic Parameters of Pg Activation by t-PA in the Presence of ECM-Associated Proteins[a]

protein	K_m (μM)		V_{max} ($\times 10^{11}$ mol of Pm/min)		k_{cat} (s^{-1})		k_{cat}/K_m (μM^{-1} s^{-1})		x-fold k_{cat}/K_m increase	
	Glu	Lys	Glu	Lys	Glu	Lys	Glu	Lys	Glu	Lys
0	0.13	0.06	0.48	0.26	0.15	0.08	1.15	1.33		
Fbg	0.11	0.05	2.50	1.73	0.76	0.52	6.91	10.40	6.0	7.8
CNBrFbg	0.17	0.04	6.53	1.90	1.98	0.58	11.65	14.50	10.1	10.9
coll I	0.04	0.42	1.14	1.70	0.35	0.52	8.75	1.24	7.6	0.9
gel. I	0.02	0.06	0.83	1.52	0.25	0.46	12.50	7.67	10.9	5.8
coll III	0.10	0.15	1.71	2.43	0.52	0.74	5.20	4.93	4.5	3.7
gel. III	0.13	0.05	0.52	2.02	0.16	0.61	1.23	12.20	1.1	9.2
coll IV	0.02	0.07	1.58	1.23	0.48	0.37	24.00	5.29	20.9	4.0
gel. IV	0.01	0.02	2.07	1.00	0.63	0.30	63.00	15.00	54.8	11.3
coll V	0.04	0.05	1.50	2.45	0.45	0.74	11.25	14.80	9.8	11.1
gel. V	0.02	0.07	1.05	3.62	0.32	1.10	16.00	15.71	13.9	11.8
FN	0.54	0.09	1.05	1.63	0.32	0.49	0.59	5.44	0.5	4.1
LN	0.09	0.04	1.77	2.17	0.54	0.66	6.00	16.50	5.2	12.4
BSA	0.09	*b*	0.66	*b*	0.20		2.22		1.9	

[a] Abbreviations: Fbg, fibrinogen; coll, collagen; gel., gelatin. [b] Does not follow Michaelis–Menten kinetics.

Table II: Effect of ECM Proteins on t-PA Amidolytic Activity

protein	velocity ($\times 10^{11}$ mol of pNA released/min)	x-fold increase
0	2.2	
Fbg	23.0	10.5
CNBrFbg	16.1	7.3
coll I	7.0	3.2
coll III	6.6	3.0
coll IV	5.0	2.3
coll V	7.7	3.5
gel. I	13.0	5.9
gel. III	9.7	4.4
gel. IV	12.8	5.8
gel. V	12.3	5.6
FN	26.4	12.0
LN	16.4	7.3
BSA	5.0	2.3

Table III: Effect of ECM Proteins on Plasmin Amidolytic Activity

protein	velocity ($\times 10^{10}$ mol of pNA released/min)	x-fold increase
0	6.5	
Fbg	8.1	1.2
CNBrFbg	1.5	0.2
coll I	12.8	2.0
coll III	7.6	1.2
coll IV	8.9	1.4
coll V	9.5	1.5
gel. I	8.4	1.3
gel. III	6.4	1.0
gel. IV	9.0	1.4
gel. V	8.8	1.4
FN	6.6	1.0
LN	9.5	1.5
BSA	7.0	1.1

tivation of Lys_{77}-Pg in the presence of BSA did not follow Michaelis–Menten kinetics. Under all other conditions, activation of Glu and Lys_{77}-Pg obeyed Michaelis–Menten kinetics. Kinetic parameters for activation of Glu-Pg and Lys_{77}-Pg are summarized in Table I. Activation of both Glu-Pg and Lys_{77}-Pg was stimulated under a large variety of conditions. With Glu-Pg as substrate, preincubation with many protein components resulted in a decrease in K_m as well as an increase in V_{max}. However, with Lys-Pg, effects on V_{max} are more prevalent. Table I also illustrates the increase in catalytic efficiency (k_{cat}/K_m) of Pg activation in the presence of various protein components.

Effect of Protein Components on Pm Amidolytic Activity. The effect of ECM-associated proteins on the amidolytic activity of Pm generated in the activation reaction was studied by using VLK-pNA. Table II demonstrates that a slight enhancement (1.2–2.0-fold) in Pm activity was obtained in the presence of ECM protein components. This effect appears to be nonspecific, however, since a similar enhancement was observed in the presence of BSA. Inhibition of Pm amidolytic activity was observed in the presence of CNBr fibrinogen fragments and may represent competition of the fragments for the active site of Pm.

Effect of Protein Components on t-PA Amidolytic Activity. To determine whether the increased catalytic efficiency observed in the presence of ECM-associated proteins was attributable solely to an increase in t-PA activity, the amidolytic activity of t-PA was assayed in the presence of protein components. Addition of collagen resulted in a slight increase in t-PA activity similar to that observed with BSA (Table III). Gelatins were better stimulators than the corresponding collagen types, resulting in a 4.4–5.9-fold increase in t-PA activity. The presence of LN or FN caused an increase in t-PA amidolytic activity comparable to that observed with fibrinogen or CNBr fibrinogen fragments.

Discussion

Previous studies in a number of laboratories have demonstrated that Pg activation by t-PA is enhanced by the presence of fibrin (Ranby, 1982) or fibrinogen fragments (Nieuwenheuizen, 1983). Kinetic data indicate that a ternary complex is formed between fibrin, Pg, and t-PA, thereby enhancing the efficiency of the activation reaction (Hoyalerts, 1982). Several recent reports have shown that Pg and t-PA also interact with macromolecules unrelated to fibrin. Salonen et al. (1985) demonstrated that Lys_{77}-Pg and t-PA are bound by FN. Physiological concentrations of FN were shown to enhance activation of Lys_{77}-Pg, but not Glu-Pg, by t-PA and stimulate the amidolytic activity of Pm and t-PA (Gilboa & Kaplan, 1985). tPA and Glu-Pg can also bind to TSP or HRGP, resulting in increased efficiency of Pm generation (Silverstein et al., 1985). In addition, activation of Glu-Pg is stimulated following binding to extracellular matrices synthesized by endothelial cells (Knudsen et al., 1986). Together these studies indicate that activation of Pg by t-PA may be a widespread mechanism for generation of localized extracellular proteolytic activity.

In this study, the kinetics of activation of Glu-Pg and Lys_{77}-Pg (isoform 2) by t-PA were determined in the presence of various protein components of the ECM. Previous studies characterizing the effect of stimulators such as fibrinogen on Pg activation were performed with a mixture of Pg isoforms

1 and 2 as well as in the presence of components such as Cl^- or gelatin which themselves influence Pg activation kinetics (deSerrano et al., 1989). Separation of Pg isoforms prior to kinetic determinations is essential since it has been demonstrated that plasminogens 1 and 2 differ in many respects including carbohydrate content (Hayes & Castellino, 1979), activation kinetics (Takada et al., 1985), and receptor binding (Gonzalez-Gronow et al., 1989). Since Glu-Pg is readily converted to Lys_{77}-Pg by limited proteolysis (Castellino, 1981), the effect of ECM proteins on Lys_{77}-Pg activation was also determined.

The kinetic data demonstrate that, with the exception of FN, all of the ECM protein components tested stimulated the activation of both Glu and Lys-Pg by t-PA (Table I). In general, increased efficiency of Glu-Pg activation resulted from both a decrease in K_m and an increase in k_{cat}. With Lys-Pg, however, K_m changes were minor compared to relatively large increases in k_{cat}. Glu-Pg undergoes large conformational changes following ligand binding which may alter its efficacy as a t-PA substrate and thereby alter K_m values (Castellino, 1981). Although the presence of ECM proteins had little effect on the amidolytic activity of Pm (Table II), t-PA amidolytic activity was significantly enhanced by gelatin, FN, and laminin (Table III).

To determine the effect of collagenous components of the ECM on Pg activation, Glu-Pg and Lys_{77}-Pg were activated by t-PA in the presence of collagen types I, III, IV, and V. Type I, the most abundant collagen in the body, is located in skin, tendon, and bone and is usually accompanied by varying quantities of collagen III (Kuhn, 1987). The macromolecular structure of collagens I and III consists primarily of fibrils containing a continuous triple-helical domain. Type V collagen fibrils are smaller than those formed by types I and III and are found in a pericellular arrangement in most interstitial tissues as well as in association with collagens I and III (Fessler & Fessler, 1987). In contrast to the wide distribution of collagens I, III, and V, type IV collagen is found only in basement membranes. The type IV collagen molecules contain triple-helical segments interrupted by globular domains which give collagen IV the flexibility to assemble into sheetlike networks characteristic of basement membranes (Glanville, 1987).

All of the collagens tested stimulated Pg activation relative to controls containing no added protein or BSA. Lys_{77}-Pg activation was enhanced most effectively by collagen V. The fiber-forming collagens (types I, III, and V) had less of a stimulatory effect on Glu-Pg activation than nonfibrillar type IV collagen. The most dramatic effect on activation kinetics was observed with native and denatured type IV collagen, which stimualted Glu-Pg activation to a greater extent than CNBr fragments of fibrinogen. Interaction of Glu-Pg and/or t-PA with regions of the unique macromolecular structure of IV collagen may explain why this collagen type was a significantly better stimulator than the other collagens.

Because collagen IV is present only in the basement membrane, these data suggest that stimulation of Pg activation in the presence of collagen IV may result in an increase in localized proteolysis of basement membrane components by generated Pm. Although collagen IV is resistant to Pm cleavage, both FN and LN are degraded by Pm (Liotta et al., 1981). Pm also activates latent type IV collagenase in vitro, thereby generating an enzyme capable of degrading collagen IV (Reich et al., 1988). In addition, metastatic tumor cells produce increased amounts of both plasminogen activators and latent type IV collagenase during basement membrane invasion in vivo (Reich et al., 1988). It is interesting to speculate that modulation of Pg activation by collagen IV may reflect an important regulatory mechanism for basement membrane proteolysis associated with tissue remodeling, cell migration, and neoplasia.

Denatured collagens (gelatins) were also potent stimulators of activation of both Glu and Lys_{77}-Pg. In particular, Glu-Pg activation was stimulated by type IV gelatin to a much greater extent than by any other protein including fibrinogen fragments. t-PA amidolytic activity was enhanced approximately 5-fold, while no increase in Pm amidolytic activity was observed. Denaturation of collagen may occur under physiological conditions of temperature and pH due to destabilization of the collagen triple helix following collagenase cleavage. Previous studies have demonstrated the ability of a number of denatured proteins to stimulate Pg activation. deSerrano et al. (1989) presented data indicating that the stimulatory effect of fibrin on t-PA activation of Pg in vitro is due at least in part to denaturation of fibrin during the solubilization process. Acid- or base-denatured proteins such as immunoglobulin G and ovalbumin also stimulated the activation of Pg by pig heart activator (Radcliffe & Heinze, 1981). These data indicate that interaction with denatured proteins may provide a general mechanism for enhancement of Pg activation. However, stimulation of Glu-Pg activation by type IV gelatin was much greater than that observed with other gelatins and may represent a specific interaction between Pg, t-PA, and gelatin IV.

Noncollagenous components of the ECM also modulated activation of Pg by t-PA. LN is a ubiquitous structural component of the basement membrane and is known to possess binding sites for cells as well as the triple-helical domain of collagen IV (Timpl, 1989). FN is an adhesive glycoprotein found in plasma as well as in the basement membrane (Liotta et al., 1986). Activation of Glu-Pg and Lys_{77}-Pg (k_{cat}/K_m) was stimulated 5.2- and 12.4-fold, respectively, in the presence of LN. LN also enhanced t-PA amidolytic activity 7-fold with no effect on Pm activity. FN was the most potent stimulator of t-PA amidolytic activity (12-fold) with no effect on Pm activity. A differential effect on the activation of Glu-Pg and Lys_{77}-Pg by t-PA was observed in the presence of FN. Activation of Glu-Pg was inhibited by FN whereas Lys_{77}-Pg activation was stimulated 4-fold. Similar results were reported by Gilboa and Kaplan (1985) using a mixture of Pg isoforms 1 and 2. The inhibitory effect observed with Glu-Pg in this study was due primarily to a large increase in K_m, suggesting that in the presence of FN, Glu-Pg adopts a conformation that is not readily bound by t-PA. The differential effect of FN on Glu-Pg and Lys_{77}-Pg activation may also be related to the observation that t-PA and Lys_{77}-Pg bind FN with much greater affinity than Glu-Pg (Salonen et al., 1985). In summary, this study demonstrates that several protein components of the ECM, most notably type IV collagen, enhance the activation of Pg by t-PA. Current models for regulation of physiological fibrinolysis suggest that Pg activation is a surface-associated process. Assembly of Pg and t-PA on a fibrin clot or cell surface results in increased efficiency of activation of Pg, localized Pm production, and protection of generated Pm from circulating proteinase inhibitors such as A-2-antiplasmin (Miles & Plow, 1988). Our data and those of others (Knudsen et al., 1986; Salonen et al., 1985; Silverstein et al., 1985) demonstrate that components of the ECM may provide an alternative surface for Pg activation, thereby establishing an important mechanism for localized generation of Pm activity in the ECM.

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